

# Reengineering Mexiletine by chemical synthesis to decrease toxicity and improve pharmacological properties with patient-derived iPSC cardiomyocytes

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## Abstract

Heart disease is one of the top causes of healthcare expenses in the United States. Lethal ventricular cardiac arrhythmia can arise in acquired or congenital heart disease. Long QT syndrome type 3 (LQT3) is a congenital form of ventricular arrhythmia caused by mutations in the cardiac sodium channel SCN5A. Mexiletine is a Class 1 antiarrhythmic drug that inhibits  $I_{Na-L}$  and shortens the QT interval in LQT3 patients. However, slightly above therapeutic doses, Mexiletine prolongs the cardiac action potential. Mexiletine was reengineered in an iterative process called dynamic medicinal chemistry to explore structure activity relationships (SAR) for AP shortening and prolongation of AP kinetics in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Certain newly synthesized Mexiletine analogs showed enhanced potency and selectivity for  $I_{Na-L}$  and low proarrhythmic liability and less central nervous system toxicity than the parent compound. In an aged animal model of oxidative stress that produces early after depolarization (EAD) formation and other arrhythmogenic effects, certain Mexiletine analogs examined were observed to reverse arrhythmogenic effects. Further modification using selected deuterated Mexiletine analogs showed that improvement to pharmacologic and pharmaceutical properties can be achieved. In conclusion, studies highlighted the utility of using hiPSC-cardiomyocytes to guide medicinal chemistry and obtain new chemotypes for "cardiovascular drug discovery in a dish".

**Keywords:** Human induced pluripotent stem cells (hiPSCs); Ventricular tachycardia (VT); Ventricular fibrillation (VF); Action potential (AP); Long QT syndrome type 3 (LQT3)

## Introduction

Worldwide, heart disease accounts for the greatest mortality than any other illness. In the United States, approximately one million individuals are hospitalized every year for arrhythmias, making arrhythmias one of the top causes of healthcare expenditures with a direct cost of almost \$50 billion annually for diagnosis, treatment and rehabilitation [1]. Another 300,000 individuals die of sudden arrhythmic death syndrome every year in the United States [2]. Arrhythmias are very common in older adults but unfortunately, drugs to treat arrhythmias have liabilities. In addition, numerous drugs have been withdrawn from the market because they induce QT prolongation and a potentially fatal ventricular tachycardia (VT), Torsade de Pointes (TdP) [3].

Drug development efforts to treat arrhythmias is challenging. However, recent advances in replication of human pathogenetic processes *in vitro*, using human induced pluripotent stem cell (hiPSC)-based models can facilitate development of therapeutics that target specific disease mechanisms. Thus far, large-scale drug development efforts have been restricted to using iPSCs from healthy donors [4]. hiPSC-based cell toxicity evaluation can play an important role in the development of a drug candidate and adverse cardiovascular effects of drug candidates during clinical trials that can be a major reason for termination of development of drug candidates can be clarified. Prediction of drug candidate toxicity can use *in vitro* as well as *in vivo* models. A major drawback for *in vivo* data, however, is the lack of extrapolation from small animals to humans, due to interspecies variations. With advances in stem cell technology, application of stem cell-based toxicity testing has opened up methods to study the impact of

new chemical entities not only on specific cell types, but also organs. For example, stem cell-derived three-dimensional cultures called organoids can be grown from human stem cells and from patient-derived iPSCs. These organoids have the potential to model human disease and also have potential for drug candidate testing [5,6].

Human patient-derived iPSCs can be used as a model of cell function in response to drug candidates. In addition, advances in tissue engineering, high throughput screening and microfluidics to produce disease in a dish or organ on a chip, has led to assays that are useful to understand potency as well as adverse and toxic effects of drugs to enable drug development in a dish [7]. This Commentary summarizes recent studies that describe a role of human patient-derived iPSC-derived cardiomyocytes to reengineer a cardiovascular drug with liabilities (i.e., Mexiletine) to new drug candidates. Evaluation of several toxicological endpoints will be highlighted that resulted in improved drug candidates. In the future, such an approach can be paired with human efficacy and safety studies to conduct “clinical trials in a dish” studies to support development and validation of new regulatory paradigms to assess drug safety [8].

## Methods

### Chemistry

Test compounds were synthesized and fully characterized as described previously [9-11].

### Cell culture and data acquisition

Normal and LQTS3 hiPSC-derived cardiomyocytes were prepared as described previously [12] and after 25 days cardiomyocytes were dissociated and plated onto Matrigel-coated 384 well tissue culture plates. VF2.1.C1/pluronic F127 dye mixture was loaded into cells and after equilibration, test compounds were incubated for 5 mins before image acquisition in a time series for each compound was conducted. Image analysis and physiological parameters were obtained using a IC200 KIC instrument (Vala Sciences, San Diego, CA) as described previously [13]. ADP<sub>75</sub> and cardiomyocyte beat rate was determined and statistics were applied to the data.

### In vitro metabolism

Metabolism of certain test compounds was done as described previously [9].

### In Vivo and ex Vivo Studies

Animal work followed the Guide for Care and Use of Animals as adopted by NIH. The pharmacological effect of certain Mexiletine analogs was studied in mice and previously reported [9,10]. The effect of select Mexiletine analogs on rat heart tissue isolated from aged adult Sprague Dawley rats was examined. Administration of hydrogen peroxide to perfused isolated Langendorff rat hearts showed oxidative stress (i.e., EADs and ventricular fibrillation) [14]. Treatment of these same hearts perfused with hydrogen peroxide with certain Mexiletine analogs (10 μM) showed complete resolution of arrhythmias back to normal sinus rhythm.

## Results

### In vitro studies: Antiarrhythmic drugs

While efforts in drug discovery and development efforts have increased, on a percentage basis, the number of drug approvals

has declined. One significant reason is adverse side effects of drug candidates to the heart. This has caused cessation of many preclinical drug development programs. This is partly due to lack of suitable humanized preclinical models. Human iPSC-derived cardiomyocytes have emerged as powerful non-animal tools to model heart disease, screen drug candidates, and test drug cardiotoxicity in a high-throughput and cost-effective manner [15-20]. The utility of human iPSCs include non-invasive formation and integration/correlation of patient samples with rare diseases. iPSCs have been widely used in cardiac disease modelling and the study of inherited arrhythmias. The focus of the current Commentary is to detail some *in vitro* and *in vivo* aspects of cardiovascular drug development [21].

The original classification of antiarrhythmic drugs (Classes I-VI) has remained largely empirical [22] and the link between specific ionic effect(s) of a drug and suppression of a specific arrhythmia mechanism(s) remain(s) incompletely understood [23]. Nevertheless, antiarrhythmic drugs are useful for human genetic heart disorders. In cardiomyocytes, the pathological rise of the late Na current known as late I<sub>Na</sub> (I<sub>Na-L</sub>), originates under cardiac conditions associated with increased risk of developing ventricular tachycardia (VT) or ventricular fibrillation (VF) [24] secondary to the activation of CaMKII signaling pathway [25] and mutations of Nav1.5 channels [26, 27]. Some Class I antiarrhythmic drugs such as Mexiletine block peak I<sub>Na</sub> but also display variable block of I<sub>Na-L</sub> [28]. However, the potential of proarrhythmia for patients with ischemic heart disease with these agents has led to the development of more selective blockers of I<sub>Na-L</sub>, with minimized effect on peak I<sub>Na</sub> [29].

Peak I<sub>Na</sub> occurs in less than a millisecond and underlies the rapid upstroke or phase 0 of the action potential (AP) when the activation gate and the inactivation gate on the sodium channel are both open. Over several milliseconds the current begins to decay and contributes to a notch in the AP called phase 1. Then, some of the channels are inactivated with the inactivation gate closed. There is no commonly accepted name for this phase of I<sub>Na</sub> but often it is labeled “early I<sub>Na</sub>.” After several milliseconds I<sub>Na</sub> normally decays to <1% of peak I<sub>Na</sub>. A residual current flow as I<sub>Na-L</sub> and this depolarizing current along with calcium currents supports phase 2 or the plateau of the AP. The mechanisms for late I<sub>Na-L</sub> at the sodium channel level are multiple but can generally be thought of as incomplete inactivation. Eventually, activating potassium currents (I<sub>Kr</sub>) repolarize the membrane (phase 3 of the AP) and when the voltage decreases below the sodium channel threshold, the activation gate closes.

Current classes of antiarrhythmic drugs are not capable of selective discrimination between the pathological I<sub>Na-L</sub> and the normal peak I<sub>Na</sub> necessary for normal AP upstroke velocity because often, they suppress both the early and late components of the I<sub>Na</sub> to an equal extent. For example, Mexiletine, a Class 1B antiarrhythmic drug has similar effects on peak I<sub>Na</sub> and I<sub>Na-L</sub>. Arrhythmias can cause a multitude of chronic cardiac diseases including heart failure [30], myocardial ischemia [29], increased pro-oxidant states [31-33], and hypertrophic cardiomyopathy [24,34]. Abnormal cardiac physiology can arise from pathological I<sub>Na-L</sub>. For example, early after depolarizations (EADs) and EAD-mediated arrhythmia can originate from cardiomyocytes. The potent sea anemone toxin II (ATX-II) can be used as a pharmacological tool to selectively enhance cardiomyocyte I<sub>Na-L</sub> with minimal effect on other ionic currents [35,24]. The selective and potent blocker (i.e., IC<sub>50</sub> of 134 nM) of the I<sub>Na-L</sub> (i.e., GS-458967) that works as a ‘gating modifier’, showed its role in decreasing EAD-mediated arrhythmias and suppressing

$I_{Na-L}$ . This is fundamentally distinct from the traditional Class 1 antiarrhythmic drugs like Mexiletine that block the peak  $I_{Na}$  channel.

### Long QT syndrome-3 (LQT3)

Long QT syndrome-3 (LQT3) is characterized by abnormal heart beats caused by mutations in the sodium channel  $\alpha$  subunit [36]. Cardiomyocytes encoding mutant sodium channels that predisposes a patient to VT and sudden death can afford a genetic congenital model of LQTS3 [37]. The cardiac conditions associated with increased risk of VT/VF include human congenital LQT3 [26, 27]. Certain patients are genetically predisposed to a potentially fatal arrhythmogenic response to Mexiletine to treat LQT3 because the drug has off-target effects on other ion channels including the hERG channel [38]. hERG conducts the rapidly activating component of the delayed rectifier potassium current ( $I_{Kr}$ ) that is responsible for the bulk of ventricular repolarization in cardiomyocytes. Mexiletine also has minor but detectable adverse drug properties including hepatotoxicity and blood dyscrasias [36]. In addition, Mexiletine suffers from a relatively short half-life that necessitates frequent doses. Additional doses of Mexiletine can elicit CNS toxicity.

Development of potent and selective blockers of late sodium currents could offer a new and effective antiarrhythmic drug class. Below, Mexiletine analogs are described that show considerable selectivity against late  $I_{Na-L}$  without affecting their peak response [12]. Mexiletine is used to shorten QTc in LQT3 patients in the hope that this shortening will decrease the probability of lethal arrhythmias. The idea of antiarrhythmic drug therapy based on changes in ion channel gating rather than peak current block (i.e., gating modifiers) could provide a new class of drug candidates [39].

### Ex vivo studies

Oxidative activation of calcium/calmodulin dependent protein kinase II (CaMKII) by hydrogen peroxide ( $H_2O_2$ ) increases the  $I_{Na-L}$  and promotes EADs in isolated rat and rabbit ventricular myocytes *in vitro* [31-33]. This can be translated into an *ex vivo* model because EAD formation and other arrhythmogenic effects can be observed after administration of  $H_2O_2$  to perfused Langendorff rat and rabbit hearts [14]. Oxidative stress has been shown to induce EADs in isolated myocytes in these two species. However, *in vivo*, perfusion of  $H_2O_2$  failed to induce ventricular arrhythmias in normal healthy young rat or rabbit hearts [40]. In contrast, aged rat hearts (i.e., 24-26 months old rat hearts), showed increases in tissue fibrosis (10-90%) and decreased cell-to-cell gap junctional couplings. Perfusion of aged rat hearts with  $H_2O_2$  consistently promoted EADs and led to an incidence of >90% of aged fibrotic hearts to tachycardia and fibrillation (VT/VF) [14].

A black box warning for Mexiletine (Class I) by the FDA came about after the CAST clinical trial showed that inhibition of peak Na current was ineffective and even carried greater risk of mortality [41]. It should be pointed out the black box warning followed the CAST study was based on post-myocardial infarction patients and not LQT3 patients. Later, the SWORD clinical trial with d-sotalol (Class III) [38] was done based on the idea of increasing cardiac refractoriness. Results of the CAST and SWORD studies showed drugs with Class I and Class III actions are ineffective in suppressing arrhythmia following myocardial infarction [41,42].

### Case study: Development of mexiletine analogs for LQT3

As described above, LQT3 is characterized by abnormal heart

beats caused by mutations in the  $Na^+$  channel [36]. Except for one publication [43], evidence for proarrhythmic effects of Mexiletine in LQT3 patients has not been reported. Mexiletine is used to shorten QTc in LQT3 patients in the hope that this shortening will decrease the probability of lethal arrhythmias. However, Mexiletine has off-target effects on other ion channels including the hERG channel [44-46]. Because Mexiletine possesses adverse properties, a goal of our work was to chemically re-engineer Mexiletine to afford new compounds with superior pharmacological and/or pharmaceutical properties. Development of compounds with increased on-target (i.e.,  $I_{Na-L}$ ) versus off-target  $I_{Kr}$  (i.e., hERG) selectivity was another goal. hERG inhibition is known to be associated with clinically relevant arrhythmias and EADs. Drug-induced production of EADs manifested by T-wave prolongation and premature ventricular contractions (PVCs) or measurement of cessation of cardiomyocyte beating was used as a measure of cardiomyocyte toxicity.

### Use of hiPSCs to chemically reengineer mexiletine

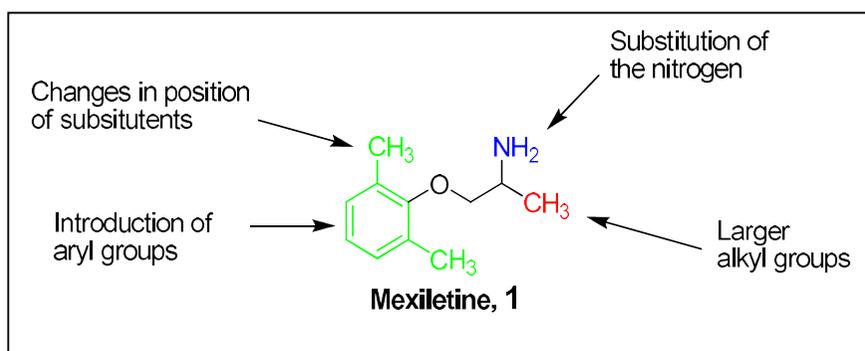
Normal and patient-derived hiPSCs are a useful source to create human cardiomyocytes to evaluate synthetic or other drug candidates in a drug development campaign. In our studies [9,12], iterative dynamic medicinal chemistry was done on a practical time scale and in 384-well multi-well format to test drug candidates. Patient iPSC-derived cardiomyocytes were used in a disease-in-a-dish approach to address an important human disease, namely QT prolongation and potentially fatal VT and TdP [9,12].

Patient-derived hiPSCs cardiomyocytes that encoded mutant sodium channels that predisposed a patient to VT and sudden death afforded a genetic congenital model of LQTS3 [47]. Normal hiPSCs cardiomyocytes were used in parallel as a control. A goal was to provide a way to identify new synthetic Mexiletine analogs to reverse a pathogenic disease phenotype. Studies conducted in parallel with normal hiPSCs cardiomyocytes were also useful to identify compounds that could potentially be toxic to normal cardiomyocytes. Identification of drug candidates that were toxic to cardiomyocytes could provide insight to structural aspects of drug candidates that caused toxicity. Molecules known to cause hERG inhibition and afford clinically relevant arrhythmia were used to validate dose-dependent drug induction of toxicity [12]. We used molecules known to cause EADs as controls and observed dose-dependent PVCs to further validate the test system [12].

### Mexiletine chemical reengineering

Mexiletine, 1 was re-engineered by chemical synthesis to afford analogs with increased on-target potency (i.e., sodium channel) and decreased off-target (i.e., potassium channel) effects [9]. The structure-activity relationship (SAR) for the effect of Mexiletine analogs on normal and hiPSC patient-derived cardiomyocytes was systematically evaluated in cardiomyocytes derived from an LQT3 patient (carrying the SCN5A F1473 mutation) and from an unrelated healthy donor [47]. Thus, the effects of Mexiletine analogs on cardiomyocytes with channelopathies (i.e., disease in a dish) were compared to the effects of compounds on normal non-pathogenic cardiomyocytes. To quantify the effect of modification of Mexiletine, the molecule was conceptually divided into three exploratory regions (Figure 1): (I) the region alpha to the primary amine moiety, (red), (II) the phenoxy region, (green) and (III) the N-substituted region, (blue).

Each compound was tested in dose-response studies in patient-



**Figure 1:** Regions of Mexiletine chemically modified: Region I is alpha to the primary amine group, Region II is the phenoxy moiety (aryl group), Region III is substitution of the nitrogen.

derived hiPSC-CMs to determine a concentration of cessation of cell beating, an EC<sub>50</sub> for shortening the action potential duration (APD), a fold-shortening of the APD and a concentration that caused shortening of the action potential. In normal cardiomyocytes, the concentration that caused cessation of cell beating, the concentration that caused pro-arrhythmic induction of EADs and the EC<sub>50</sub> for shortening the APD were determined. In total, approximately 130 compounds were synthesized and tested [9]. Structural modifications of the portion of Mexiletine alpha to the primary amine (i.e., Region I) showed that an alpha aryl moiety showed greater fold-shortening than aliphatic substituents. However, partial aryl character (i.e., cyclopropyl compounds) were less effective at fold-shortening than aryl compounds. Modifications of the phenoxy moiety (i.e., Region II) resulted in compounds with potent APD shortening. *N*-Modification of selected compounds (Region III) yielded additional derivatives with potent fold-shortening and in some cases, less toxicity to cardiomyocytes. We also tested selected Mexiletine analogs in the aged rat heart perfusion model described above. Administration of H<sub>2</sub>O<sub>2</sub> consistently promoted EADs and led to an incidence of aged fibrotic hearts to tachycardia and fibrillation. After administration to aged fibrotic hearts perfused with H<sub>2</sub>O<sub>2</sub>, certain Mexiletine analogs abrogated the EADs and returned the heart back to normal rhythm [9].

#### Deuterated analogs of mexiletine analogs

In *in vitro* metabolic studies, we observed the flavin-containing monooxygenase (FMO) [48] metabolized Mexiletine and analogs [10]. Surmising that metabolism was at the amine moiety, we incorporated a deuterium at the alpha carbon. Compared to non-deuterated compounds, incorporation of deuterium into phenyl Mexiletine analogs did not significantly alter the cardiovascular properties of the molecule but alpha deuteration of phenyl mexiletine and analogs improved pharmaceutical properties [10].

For certain Mexiletine analogs, alpha amino deuteration showed a significant increase in area under the curve (AUC) after administration to rats [10]. Development of drug analogs of Mexiletine with greater AUC and C<sub>max</sub> may allow fewer doses to patients. Frequent dosing and compliance with dosing is a major detriment to use of Mexiletine, especially in children. Also, compared to Mexiletine, side effects of seizures were observed decreased in phenyl Mexiletine classes of compound analogs examined due to

the nature of the chemical structure. Phenyl mexiletines tested were observed to be considerably less CNS toxic compared to Mexiletine [9,10]. Improved pharmacokinetic properties of phenyl Mexiletines (e.g., deuterated phenyl Mexiletines) may afford the use of lower doses and be associated with less adverse drug interactions.

#### Pyridine analogs of mexiletine or phenyl mexiletine

We synthesized and tested approximately 30 pyridyl analogs of Mexiletine or phenyl Mexiletine [11]. For compounds examined, cell toxicity was dependent on the pyridyl substitution pattern of the compounds. For example, EADs were observed for 2-pyridyl phenyl Mexiletine and 4-pyridyl phenyl mexiletine. However, generally, from among 3-pyridyl phenyl Mexiletines tested, most did not produce EADs in normal cardiomyocytes [11]. We judged these compounds to be non-toxic because they did not cause cessation of beating or EADs in normal cardiomyocytes. Because Mexiletine can cause seizures in humans at elevated doses [49,50] examination of potential cell toxicity in human cardiomyocytes in a drug development campaign may be an efficient means of identifying untenable drug candidates.

Compared to Mexiletine, one synthetic pyridyl analog of Mexiletine was 22-fold more potent for the I<sub>Na-L</sub> channel (i.e., 1.04 versus 22.5 μM) [11]. In addition, compared to Mexiletine, the analog was almost 5-fold more selective for the sodium versus the hERG channel (i.e., IC<sub>50</sub> I<sub>Kr</sub>/IC<sub>50</sub> I<sub>Na-L</sub>=2.4 and 11.2, respectively). Thus, from this study, a pyridyl analog of Mexiletine emerged as a potent I<sub>Na-L</sub> channel inhibitor with good physicochemical properties. We concluded that synthesis and testing of pyridyl phenyl or other analogs of phenyl Mexiletine could lead to new chemotypes as potent and selective drug candidates to treat arrhythmias.

#### Conclusion

Although examples of the quantification of arrhythmia in hiPSC cardiomyocytes have been reported [51-53], including optogenetic methods [54,55], calcium sensitive probes [56] and voltage sensitive probes [56,57], our studies [9-12] were the first large scale, automated evaluation of synthetic analogs from iterative dynamic medicinal chemistry using patient-derived hiPSC cardiomyocytes. The approach facilitated rapid generation of physiologically relevant parameters useful in drug development. Our study was the first to use a voltage sensitive probe to characterize a patient-specific hiPSC

cardiomyocyte disease model to show reversion of pathogenic disease phenotype with small molecule drug candidates *in vitro*. In summary, hiPSC cardiomyocyte technology in cardiovascular drug discovery and clinical management of heart disease is emerging as a viable method to test whether individual patient differences can predict clinical outcome.

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